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13. ABSTRACT ***(Maximum 200 Words)***

Epidemiological evidence and studies in cancer models suggest that dietary plant estrogens (phytoestrogens) reduce the risk of breast cancer. In this work we examined the role of phytoestrogens as anti-cancer drugs by inhibiting cell proliferation through mechanisms independent of the tumour suppressor p53. This is relevant since p53 is found mutated or absent in half of the human tumors. Using p53-deficient breast cancer cell lines (BT20 and T47D), we studied the anti-proliferative effects and the mechanism of action of several phytoestrogens (biochanin, daidzein, genistein, and genistin). The main finding were: genistein and genistin seem to posses anticancer drug characteristics as suggested by inhibition of cell proliferation and cell cycle arrest; G_2 arrest occurred upon treatment with phytoestrogens; cells were G_2 arrested with no p21 expression increase, thereby suggesting a p21-independent pathway for cell cycle arrest; Cdc2 activity was decreased by genistein with no changes in p21 expression. The results suggest that phytoestrogens not only have preventive effect, but can also act as anti-breast cancer drugs, by a mechanism that is independent of p53.

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INTRODUCTION

Epidemiological evidence and studies in cancer models suggest that dietary plant estrogens (phytoestrogens) reduce the risk of breast cancer. This, together with the need for development of new anticancer drugs targeting the uncontrolled cell cycle machinery, warrants vigorous research on the molecular mechanisms of action of phytoestrogens in breast cancer. The relevance and purpose of this research are summarized in these two critical issues. First, it recognizes the necessity of implementing cancer therapies involving cell cycle control that are independent of the tumor suppressor p53, since it is mutated or absent in half of the human tumors. This research is concerned specifically with the expression of cell cycle inhibitors (such as p21) by p53-independent pathways, *via* activation of transcription factors within the signal transduction cascades. Second it takes into account the major molecular and cellular mechanisms of action of phytoestrogens, such as modulation of key enzyme activities involved in signal transduction and cell proliferation and antioxidant actions.

BODY

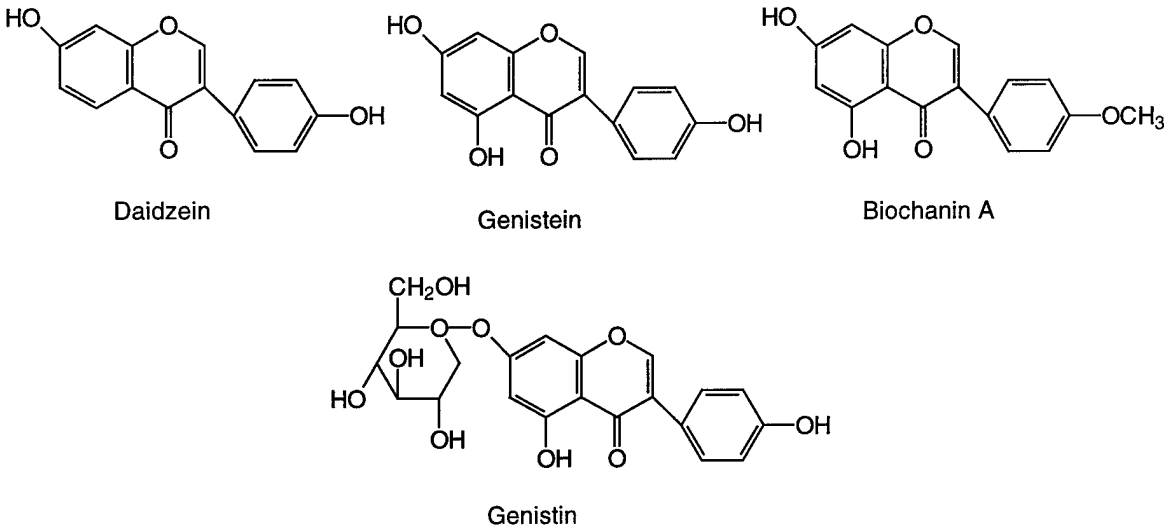
Hypothesis and Tasks— The hypothesis advanced in the original proposal and the tasks outlined in the Statement of Work remain unchanged:

- The original *hypothesis* sustained that cellular redox changes elicited by phytoestrogen actions in breast cancer cells lead to disruption of the cell cycle upon induction of the negative cell cycle regulator p21 *via* activation of signal transduction cascades.
- The tasks referred to in the Statement of Work were aimed at proving this hypothesis:
  - *Task 1.* To identify and characterize the changes in redox status in breast cancer cells in response to phytoestrogen action.
  - *Task 2*: To identify the molecular mechanisms involved in the phytoestrogen-mediated expression of inhibitors of the cell cycle.
  - *Task 3*: To identify phytoestrogen-mediated, redox-sensitive, p21-dependent pathways leading to inhibition of cell proliferation.

Research Findings — Phytoestrogens are a diverse group of substances that have a chemical structure similar to that of steroidal estrogens. Research during the period for this annual report focused primarily on isoflavones, one of the dominant classes of estrogenic substances found in plants. The effect(s) of selected phytoestrogens were examined in terms of their ability to inhibit cell proliferation, cell cycle arrest, induce or not apoptosis, and activation of cyclins in a variety of cells.

- *Materials and Methods* — Cells used in these studies are listed in Table I in terms of their

p53 content or functionality and the presence or absence of an estrogen receptor. During this period, BT20 and T47D were the cell lines examined. Breast cancer cells BT20($p53^{-/-}$) and T47D($p53^{-/-}$) were grown in Minimum Essential Medium (MEM) and RPMI Medium 1640 (Gibco BRL), respectively, with 10% fetal calf serum, and 1% penicillin-streptomycin (Gibco BRL). Cells numbering 1×10^6 were seeded on 100-mm diameter dishes 24 hours before drug treatment. Control cultures were treated with DMSO alone. Cell numbers were counted in hemacytometer by light microscopy. Genistein, genistin, biochanin A, daidzein, and diadzin were purchased from Sigma, dissolved in dimethyl sulfoxide (DMSO) and diluted to the respective final concentrations in each culture dish. Apoptotic cells were identified using the Annexin V-FITC Apoptosis Detection Kit



Scheme I. Structures of the phytoestrogens used in this work.

(Calbiochem). The protocol for Annexin V Binding with Adherent Cells was used followed by Rapid Annexin V Binding protocol according to manufacture instructions. Annexin V staining was used with flow cytometry to detect apoptotic cells. Cell cycle analysis (1): DNA content *per* duplicate was analyzed using FAC-Star flow cytometer (Becton Dickinson, Mountainview, Ca.) according to established procedures. Forty-eight hours after the addition of the isoflavone, cells were removed from the culture dish by trypsinization, washed with PBS and fixed in 70% ethanol and kept at 4°C until analysis. Cells were stained with 20*u*g/ml propidium iodide containing 20*u*g/ml RNase(DNase free) overnight. The stained cells were analyzed by flow cytometry. The populations of G_0/G_1, S, and G_2/M were quantitated using MacCycle software (2) (Phoenix Flow System, Inc., San Diego, CA.). Approximately 10^4 cells were examined in each analysis. For cell proliferation assay, experiments were run in 96-well plates. Each well contained 5×10^3 to 10×10^3

cells depending on the proliferation rate of the cell. Cell growth was determined using the Cell Titer 96 Non-Radioactive Cell Proliferation Assay Kit (Promega). The "0" point represents number of cells before drug treatment. For western blot analysis, after the appropriate incubation of the particular isoflavone, the cells were harvested, lysed with RIPA lysis buffer. Protein concentration was determined by BCA protein assay (Pierce). 30 μg of total protein from each sample were run on 12% SDS-polyacrylamide gels and blotted onto a nitrocellulose filter. The filter was blocked with TBS tween 0.1% containing 5% of dry milk, than incubated overnight with polyclonal $p21^{Waf}$ or cdc2 antibody, which was diluted in 5% milk/TBS tween. TBS tween 0.1% was than used to wash the nitrocellulose. Detection was achieved using the Pierce Supersignal West Pico Kit, with 1min and 5min exposure times. After immunoprecipitation cyclin-dependent kinase cdc2 activty was measure with a histone H_1 kinase assay as described before (3).

- *Results* — The phytoestrogens were screened by their ability to cause cell cycle arrest. Different concentrations were used for 48-hour phytoestrogen treatments. Among the phytoestrogens tested, genistein and its glucoside genistin exerted the more significant effects on cell cycle parameters (Table 2): at the concentration of 25 - 100 μM they caused a large increase in the percentage of cells in G_2 in the BT20($p53^{-/-}$) and T47D($p53^{-/-}$) breast cancer cell lines. An increase in the percentage of cells in G_2 does not necessarily mean that G_2 arrest is actually occurring, because the phytoestrogen treatment maybe merely slowing the G_2/M transition rate. To insure G_2 arrest was occurring, cell proliferation was determined with and without phytoestrogen treatment (Figure 1). Genistein caused inhibition of cell proliferation in both BT20 and T47D, while genistin caused cell cycle arrest only in BT20 cell line. The data suggests that genistin did not cause G_2 arrest in the T47D cell line but merely decreased the G_2/M transition rate because at 72 hours, proliferation of the T47D cells, treated with genistin, increased significantly.

  Apoptosis may also play a role in the phytoestrogen anti-proliferative effect. Incubations of genistein and genistin at 100 μM for 48 hours and 96-hour incubation induce only modest levels of apoptosis (below 20%, Table 3). If these phytoestrogens did cause apoptosis it would have been clearly evident after 96 hours. The small percentage is probably caused by the G_2 arrest. Usually when a cell's cycle is arrested for a long period of time the cell will eventually go into apoptosis.

  The next step was to determine how the phytoestrogen treatment was causing G_2 arrest. Normally anticancer drugs upregulate the expression of the cyclin-dependent kinase inhibitor, p21, to elicit cell cycle arrest. The expression of the p21 protein was determined by western analysis, but the blot showed very little to no increase of the p21 protein expres-

sion (Figure 2). Since the p21 was not upregulated, we analyzed the activity and protein levels of the cyclin-dependent kinase cdc2, because cdc2 is a critical kinase in the G2/M transition. Cdc2 activity was decreased in both BT20 and T47D cells by genistein, although the activity in T47D cells was more affected than in BT20 cells (Figure 3), which may explain why this cell line is more sensitive than the BT20 (4). The western analysis for the protein levels of cdc2 confirmed these results. In T47D cells cdc2 levels were significantly decreased, while in BT20 cells no differences were detected within the sensitivity of the western analysis (Figure 4). Overall the results indicate that genistein inhibits cyclin-dependent kinase cdc2 activity in both BT20 and T47D cells, by a mechanism that in T47D cells involves decreased levels of the cdc2 protein.

KEY RESEARCH ACCOMPLISHMENTS

- Genistein and genistin seem to posses anticancer drug characteristics as suggested by inhibition of cell proliferation and cell cycle arrest.
- G_2 arrest occurred in p53-deficient cell lines (BT20 and T47D) upon treatment with phytoestrogens.
- G_2 arrest with no p21 expression increase, thereby suggesting a p21-independent pathway for cell cycle arrest.
- Both compounds increase the total % of apoptosis, albeit to a lower extent that other apoptotic situations, such as oxidative stress.
- Cdc2 activity was decreased by genistein with no changes in p21 expression.

REPORTABLE OUTCOMES

CONCLUSIONS

Among the phytoestrogens examined, genistein and genistin show the highest potential as anticancer drugs. Both phytoestrogens, depending on the cell line, display an antiproliferative effect by causing G_2 arrest and a small amount of apoptosis. These results are not dependent on an intact or functional p53 and western analysis suggests –although not unequivocally–, that these effects are also independent of p21 expression. Hence, these isoflavones may prove to be drugs that not only prevent breast cancer but also that have promise in terms of therapeutic potential.

The actual mechanism of G_2 arrest described here requires further understanding. Usually

p21 plays a role in cell cycle arrest (independent or dependent on p53 pathways) but these data suggest that phytoestrogens may elicit this effect in a p21-independent manner. Of course, these results do not rule out the expression of other p21 family members, which maybe upregulated and cause G_2 arrest. Three factors need be considered when addressing cell cycle arrest:

a phosphorylation cascades

b synthesis of cyclins, and

c cyclin dependent kinase activity.

Genistein is known as a protein tyrosine kinase inhibitor and, accordingly, may cause cell cycle arrest by perturbing the process of phosphorylation/dephosphorylation of the tyrosine residues of *cdc*2 kinase, which is essential for cells to leave G_2 and enter M phase (5). Recent data supports the view that phytoestrogens can modulate the synthesis of cyclins but this modulation occurs concomitantly with a modulation of p21 expression (6,7). The hypothesis that phytoestrogens modulate the synthesis of cyclins independently of p21 should be examined, taking in consideration that we found that cdc2 kinase activity is inhibited, independent of p21 expression.

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APPENDICES

Table 1.

CANCER CELLS USED IN THIS STUDY

	Cell Type			
	BT20	T47D	MCF7	MDAMB23
p53	-	-	+	+
ER	-	-	+	-

"-", denotes absence of a protein; "+", denotes presence of a functional protein; ER, estrogen receptor.

Table 2. MODULATION OF CELL CYCLE PARAMETERS IN BT20 AND T47D CELLS UPON INCUBATION WITH PHYTOESTROGENS

Treatment	*Cell Type*					
	BT20			*T47D*		
	G1 %	G2/M %	S%	G1 %	G2/M %	S%
Control	55.5	22.5	21.9	43.2	44.9	11.7
Biochanin 5 μM	56.1	20.6	23.2	48.6	36.0	15.3
Biochanin 25 μM	56.1	20.6	23.2	47.1	31.0	21.8
Biochanin 100 μM	55.1	22.3	22.6	-	-	-
Daidzein 5 μM	56.2	20.4	23.2	46.3	40.6	13.0
Daidzein 25 μM	52.5	25.3	22.0	43.6	37.8	18.4
Daidzein 100 μM	51.6	30.3	18.0	-	-	-
Genistein 5 μM	56.0	20.9	23.0	50.6	30.8	18.4
Genistein 25 μM	50.2	34.4	15.3	50.5	37.3	12.1
Genistein 100 μM	21.5	52.7	25.7	30.3	69.5	0.2
Genistin 5 μM	54.6	23.3	21.9	46.5	40.4	12.9
Genistin 25 μM	47.1	29.5	23.3	13.1	75.8	11.0
Genistin 100 μM	38.4	43.8	17.7	-	-	-

Cells (1×10^6) were seeded and allowed to grow for 24 h, then treated with each phytoestrogen for 48 h. Cell cycle was measured as indicated in Materials and Methods.

Table 3. INDUCTION OF APOPTOSIS IN BT20 AND T47D CELLS UPON INCUBATION WITH GENISTEIN (100 μM) OR GENISTIN (100 μM).

	Cell Type	
Treatment	*BT20*	*T47D*
Control	1.6 %	1.6 %
Genistein (48 h)	9.8 %	1.8 %
Genistein (96 h)	14.3%	16.3%
Genistin (48 h)	2.1 %	1.5 %
Genistin (96 h)	8.5 %	9.5 %

Cells (1×10^6) were seeded and allowed to grow for 24 h, then treated with each phytoestrogen for 48 or 96 h. Apoptosis was measured as indicated in Materials and Methods.

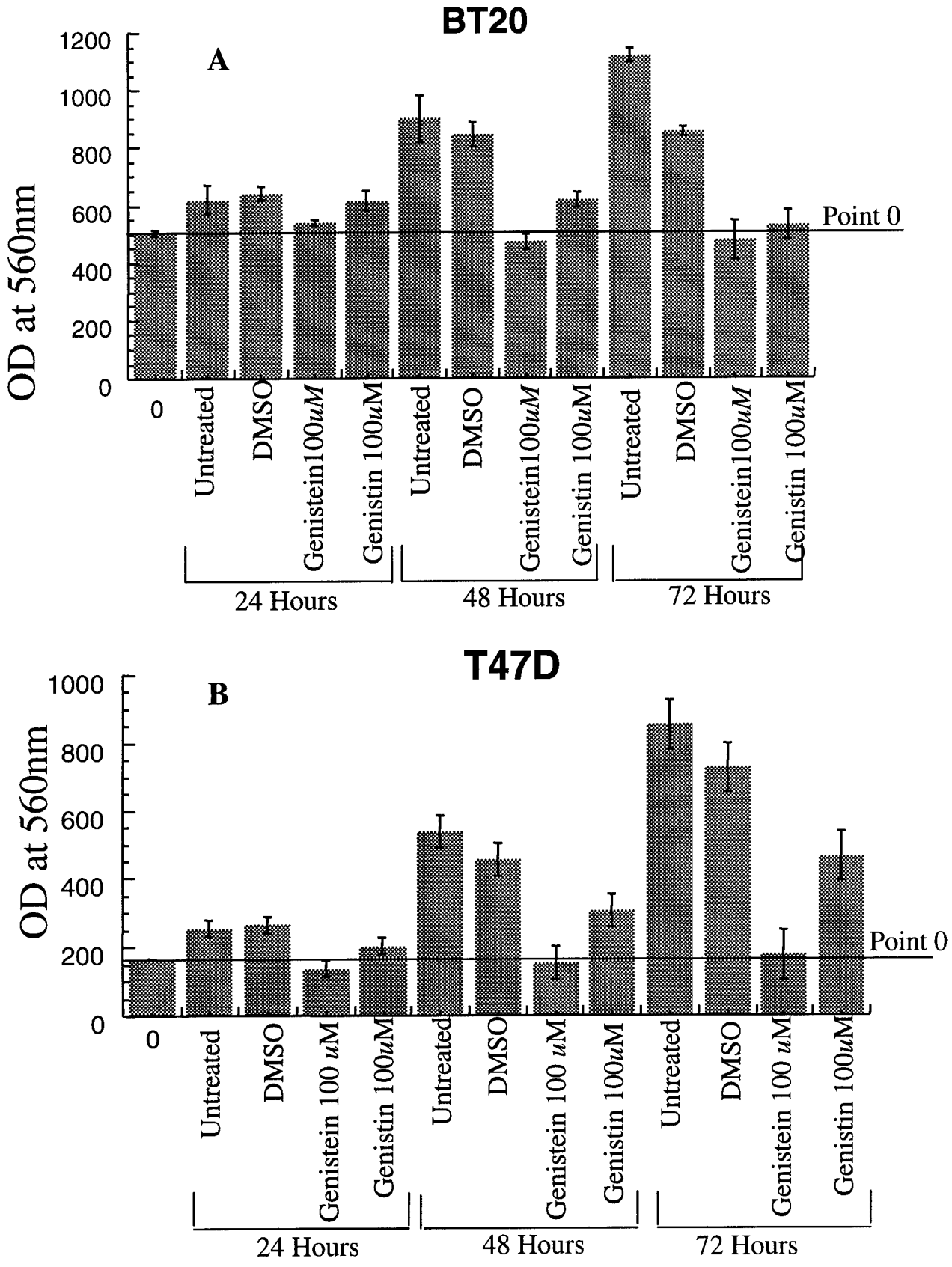

Figure 1 Cell Proliferation of BT20(A) and T47D(B) treated with genistein and genistin

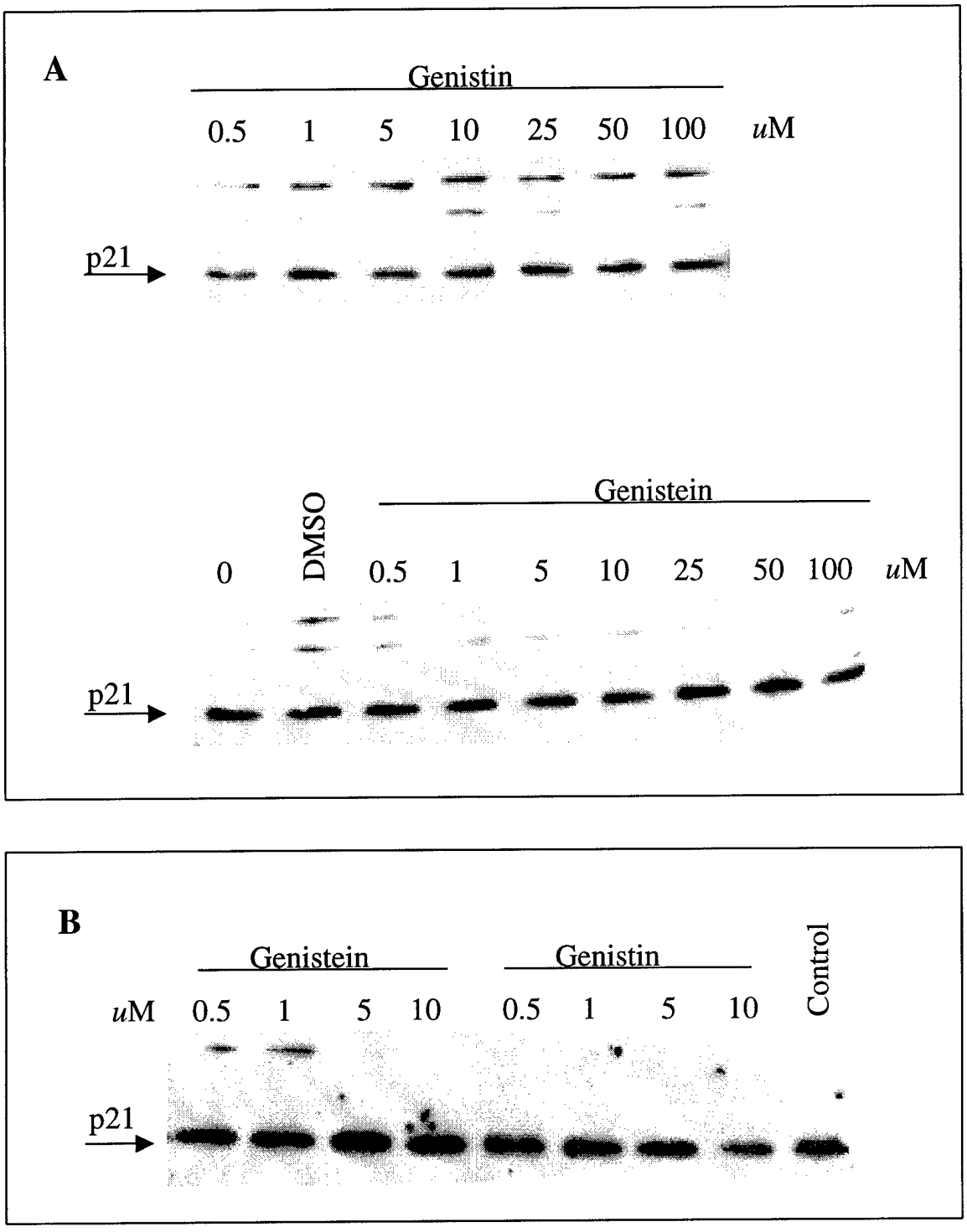

Figure 2 Western Analysis of the CDKI protein p21 of BT20(A) and T47D(B) cells treated for 8hours with genistein and genistin at different concentrations .

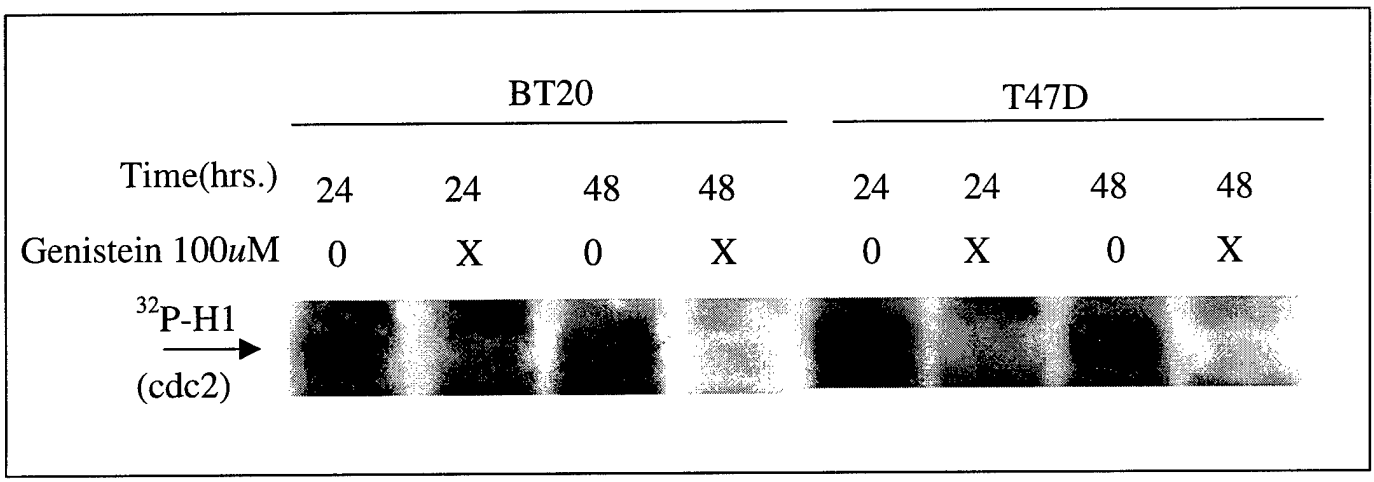

Figure 3 Effect of Genistein on cdc2 associated H1 kinase activity in BT20 and T47D cells.

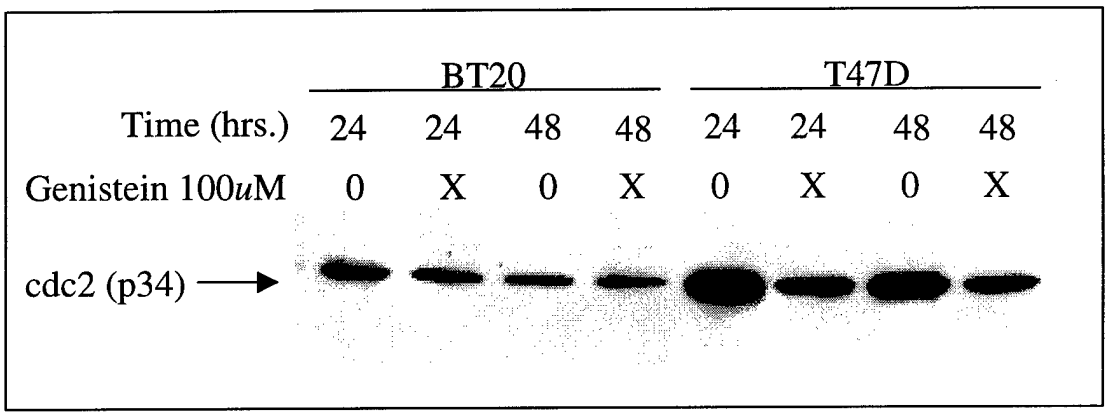

Figure 4 Western Analysis of cdc2 in the BT20 and T47D cells.